Molecular analysis of the dominant lactic acid bacteria of chickpea liquid starters and doughs and propagation of chickpea sourdoughs with selected *Weissella confusa*

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ABSTRACT

Fermented chickpea liquid is used as a leavening agent in chickpea bread production. Traditional chickpea liquid starter and dough samples were collected from bakeries in Turkey and microbiologically investigated. Culture-independent analysis for microbiota diversity, performed by MiSeq Illumina, identified *Clostridium perfringens* as major group in all samples, while *Weissella* spp. dominated LAB community. A culture-dependent methodology was applied and 141 isolates were confirmed to be members of the LAB group based on 16s rRNA gene sequence analysis. In particular, 11 different LAB species were identified

confirming the high frequency of isolation of weissellas, since *Weissella confusa* and *Weissella cibaria* constituted 47.8 and 12.4%, respectively, of total LAB isolated. The other species were *Enterococcus faecium*, *Enterococcus lactis*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Leuconostoc mesenteroides* subsp. *dextranium*, *Pediococcus acidilactici*, *Pediococcus pentosaceus* and *Streptococcus lutetiensis*. Due to high frequency of isolation, *W. confusa* strains were investigated at technological level and *W. confusa* RL1139 was used as mono-culture starter in the experimental chickpea sourdough production. Chemical and microbiological properties, as well as volatile organic compounds (VOCs) of the chickpea liquid starters and doughs were subjected to a multivariate analysis which showed the relevance of added starter, in terms of acidification and VOCs profile.

Key Words: Fermentation, cereal, legume, MySeq Illumina, RAPD-PCR, volatile organic compounds

1. Introduction

Fermentation is one of the oldest methods of food preservation and production. It improves the organoleptic properties of the foods and increases the shelf life. A diverse range of fermented foods are found worldwide and a number of them are globally distributed and produced on a large or small scale in industry, in addition to production at home (Smid and Hugenholtz, 2010; Ray and Joshi, 2015). On the other hand, some regional fermented foods are produced only in specific regions or in many regions with different cultural practices.

Chickpea bread is a traditional fermented product and is well-known in the Mediterranean and Balkan countries, especially Greece, Macedonia and some parts of Turkey. It is produced at homes and in some small-scale bakeries in the Aegean, Thrace and also some parts of the Middle Anatolia and Mediterranean Regions of Turkey. This bread type is produced by using

51 the liquid of fermented chickpea as a leavening agent (Hatzikamari et al., 2007a). For the
52 production of fermented chickpea liquid starter, coarsely ground chickpeas are put into a jar
53 or bottle and then boiled water is added. Traditional chickpea fermentation in Turkey is
54 conducted in a hot place (more than 40°C) for around 16-18 h and a thick foam layer and the
55 typical smell of chickpea liquid indicate end of the fermentation. The resulting fermented
56 liquid is then used completely for dough production or it is used after separating the chickpeas
57 in some bakeries. The chickpea dough is mixed with flour and boiled water and fermented for
58 a few hours inside the bakery and then used as a leaving agent in bread production.
59 Some studies were conducted on the application and optimization of chickpea sourdough
60 during bread production through different methods including chickpea sourdough addition or
61 using chickpea flour alone or in combination with other flours in the dough (Baykara, 2006;
62 Rizzello et al., 2014; Curiel et al., 2015; Chandra-Hioe et al., 2016; Hendek-Ertop and
63 Coskun, 2018; Shrivastava and Chakraborty, 2018). However, studies on the identification of
64 the microorganisms in chickpea fermentations are very limited.
65 In some studies, the species belong to LAB, yeasts, *Bacillus* spp. and *Clostridium* spp. were
66 identified in chickpea fermentations (Hancıoglu-Sıkılı, 2003; Hatzikamari et al., 2007b;
67 Katsaboxakis and Mallidis, 1996, Saez et al., 2018). Katsaboxakis and Mallidis (1996)
68 isolated species belonged to *Lactobacillus*, *Corynebacterium*, *Micrococcus*, *Pediococcus*,
69 *Bacillus* and *Clostridium* genera during the fermentation of coarsely ground chickpeas in
70 water. In another study conducted in Turkey, LAB species belonged to *Enterococcus*,
71 *Lactobacillus, Pediococcus, Streptococcus* and *Lactococcus* genera were identified in the
72 chickpea liquid starters and doughs collected from bakeries (Hancıoglu-Sıkılı, 2003). Cebi
73 (2009) identified the LAB strains belonged to *Lactobacillus*, *Lactococcus* and *Weissella*
74 genera in the chickpea dough samples produced using traditional procedure under laboratory
75 conditions. Erginkaya et al. (2016) counted LAB, yeasts, aerobic and anaerobic spore-forming

76 bacteria in laboratory produced chickpea dough and liquid starter sample in Turkey. Saez and
77 others (2018) identified the lactic acid bacteria species belong to four genera as *Enterococcus*
78 spp., *Lactococcus* spp., *Pediococcus* spp.and *Weissella* spp. in the chickpea sourdoughs in the
79 northwestern Argentina.

80 Identification of microorganisms isolated from spontaneous chickpea fermentations is very
81 important to design starter culture combinations since fermentation of chickpeas by starter
82 culture will enable a controlled fermentation. This is important for food industry to produce
83 those kind of fermented foods at the same quality everytime. Therefore, potentiality of the
84 industrial production of chickpea breads will be possible. For the industrial production of
85 chickpea dough, starter culture combinations can be applied and more consumers can reach
86 chickpea bread at the same quality. For strain selection, it is better to use strains isolated from
87 chickpea fermentations since they are already adapted to the conditions. Also, application of
88 starter culture in the production of chickpea sourdough at the large scale production will
89 increase production of chickpea bread and will affect consumers' diets. Industrialization of
90 chickpea bread will provide new options for the food industry and consumers. For the
91 industrial production of chickpea bread, possible starter culture combinations in dough
92 fermentation should be examined to obtain a final product with same characteristics achieved
93 under the same fermentation conditions. Therefore, the starter culture addition at industrial
94 level ensures repeatability and consistency of chickpea bread production.

95 The objective of the present study was to characterize the predominant LAB microbiota of the
96 chickpea liquid starter and dough samples collected from commercial bakeries in Turkey and
97 to select lactic acid bacterium culture showing the most relevant performances to act as starter
98 for experimental chickpea fermentations.

99

100 ## 2. Materials and Methods

101 *2.1. Sample collections*

102 The chickpea liquid starters (CLS) and chickpea doughs (CD) samples were collected from
103 three commercial bakeries, Cumhuriyet Bakery, Tokoglu Bread and Yayla Bakery located at
104 different cities in Turkey. Bakeries located at Birgi (Odemis, Izmir), Soke (Aydın) and
105 Nevsehir and the codes were assigned to the samples as A, B and N instead of using bakery
106 names due to the special request of the bakeries. Selected bakeries have been producing
107 chickpea bread for years and are well-known in their regions. A total of 12 chickpea liquid
108 starter and dough samples were sampled at two collection times between April 2016 and
109 February 2017. First and second samplings were done in the spring/summer (in the range of
110 15-20°C) and autumn/winter (in the range of 5-10°C) terms, respectively. Ambient
111 temperatures were more than 40°C in the bakeries. CLS samples were obtained by separating
112 chickpeas from the liquid inoculums at the end of the fermentation process, while dough
113 samples were collected from the final leavened doughs. All of the samples were collected into
114 sterile jars personally and reached to the laboratory within 24 hours (h) under cold conditions
115 at approximately 4˚C. Samples were analyzed immediately after reaching to the laboratory.
116 All samples were collected in duplicate.

117

118 *2.2. Preparation of the MiSeq library*

119 A 464-nucleotide sequence of the bacterial V3-V4 region (Baker et al., 2003) of the 16S
120 rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were
121 attached before the forward primers to facilitate the pooling and subsequent differentiation of
122 samples. To prevent preferential sequencing of smallest amplicons, the amplicons were
123 cleaned using the Agencourt AMPure kit (Beckman Coulter Life Sciences, USA) according to
124 manufacturer's instructions. The DNA concentration of amplicons was determined using the

125 Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In
126 order to ensure the absence of primer dimers and to assay the purity, the generated amplicon
127 libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using
128 the High Sensitivity DNA Kit (Agilent). Following quantitation, the cleaned amplicons were
129 mixed and combined in equimolar ratios. Pair-end sequencing was carried out at Genomic
130 Platform – Fondazione Edmund Mach (San Michele all'Adige, Trento, Italy) using the
131 Illumina MiSeq system (Illumina, USA).
132

133 *2.3. Illumina data analysis and sequences identification by QIIME2*

134 Raw paired-end FASTQ files were demultiplexed using idemp
135 (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative
136 Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered,
137 trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences
138 were identified and removed via the consensus method in DADA2. Representative sequences
139 were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins
140 alignment and phylogeny (Katoh and Standley, 2013; Price et al., 2009). Taxonomic and
141 compositional analysis were conducted by using plugins feature-classifier
142 (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based
143 on the Greengenes 13_8 97% Operational Taxonomic Units (OTUs) database
144 (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region
145 of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads
146 to generate taxonomy tables.
147 The data generated by Illumina sequencing were deposited in the NCBI Sequence Read
148 Archive (SRA) and are available under Ac. PRJNA 54380.
149

150 *2.4. Plate counts and LAB isolation*

151 Dough samples (10 g) were suspended with 90 mL of sterile 0.85% (wt/vol) NaCl (Merck)
152 solution in sterile stomacher bags and homogenised for 3 min at the maximum speed using a
153 bag mixer (Interscience, model 400 P, France). 10-fold dilution series of the CLS and
154 homogenised CD samples were prepared by transferring a volume of 1 mL into test tubes
155 containing 9 mL of NaCl solution. Aliquots of the decimal dilutions were spread onto
156 modified de Man Rogosa Sharpe (mMRS) (Merck) [including 1% maltose (w/v) and 5% fresh
157 yeast extract solution (v/v)] agar media to allow the growth of LAB. Incubation was
158 performed anaerobically by means of the Anaerocoult A packs (Merck 1.13829) in sealed jars
159 at 30 °C for 48-72 h. The colonies were classified according to their shape, colour, edge and
160 size, and at least 10-15 colonies per plate were picked up and purified by plate-streaking
161 technique. Presumptive LAB (Gram + and catalase -) colonies were transferred into mMRS
162 broth (Merck) containing 40% (v/v) sterile glycerol (Merck) solution and stored at -25 °C
163 until identification.

164

165 *2.5. Randomly amplified polymorphic DNA (RAPD-PCR) analysis*

166 Potential LAB isolates were subjected to genotypic characterization by RAPD-PCR analysis
167 to group the several isolates per strain. Genomic DNA was extracted using the InstaGene
168 Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.
169 Differentiation of the LAB isolates was performed using the M13 primer (Thermo Scientific)
170 following the methodology described by Gaglio et al. (2017). RAPD fragments were
171 separated using 1.2% (w/v) agarose (Sigma) gel electrophoresis prepared with 1 x TBE
172 diluted from 5 x TBE that contained 54 g/L (w/v) Trisma base (Sigma), 27.5 g/L (w/v) boric
173 acid (Merck) and 7.44 g/L (w/v) EDTA (Titriplex® III, ethylenedinitrilotetraacetic acid
174 disodium salt dihydrate, Merck). SYBR Safe^{TM} DNA gel stain (Invitrogen) was used for

175 visualization of DNA bands under UV light. 1-kb Gene ruler (Thermo Scientific) and O'Gene
176 Ruler mix (Thermo Scientific) DNA ladders were used as the molecular size markers to
177 determine the size of the amplified DNA fragment. The electrophoresis was run at 120 V and
178 then visualized (Vilber Lourmat Infinity V X 2, France) in the gel Image system. RAPD-PCR
179 profiles were analyzed using band pattern analysis employing the software package (Infinity
180 V X 2). Images of amplification fragments were scored as band absent (0) or present (1) and
181 data were entered into a binary matrix. Similarity indices of band profiles were calculated on
182 the basis of the Jaccard coefficient. Dendrograms were constructed by means of the
183 unweighted pair group method with arithmetic average (UPGMA) and one or two LAB
184 isolates of each cluster were identified by 16S rRNA gene sequencing.
185
186 *2.6. Molecular identification of LAB by 16S rRNA gene sequence analysis*
187 Molecular identification of LAB with different RAPD-PCR profiles was carried out by means
188 of 16S rRNA gene sequencing. PCR amplification was performed using primers fD1 (5'-
189 AGAGTTTGATCCTGGCTC AG-3’, Thermo Scientific) and rD1 (5'-
190 AAGGAGGTGATCCAG CC-3', Thermo Scientific) (Weisburg et al., 1991). Amplification
191 was performed in the thermocycler (Techne TC-Plus 02, UK) which was programmed as
192 follows: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 94 °C for 1 min;
193 annealing at 54 °C for 45 sec and extension at 72 °C for 2 min; plus a final extension step at
194 72 °C for 7 min.
195 PCR products were separated by electrophoresis on a 1.5% (w/v) agarose (Sigma) gel stained
196 with SYBR Safe™ DNA gel stain (Invitrogen) and subsequently visualized by Vilber
197 Lourmat Infinity (V X 2, France). PCR amplicons were sequenced at BM Laboratuvar
198 Sistemleri (Ankara). The ABI chromatograms of the sequences were examined, multiple
199 alignments were performed using ClustalW Multiple alignment (Bioedit version 7.0.9) and

then the resulting sequences were compared by Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) with nucleotide sequences deposited at the National Center for Biotechnology Information (NCBI) database (Altschul et al., 1997). LAB species identity was determined by comparison to reference sequences of the 16S rRNA gene sequences with a threshold of 98% (Yarza et al., 2014).

2.7. Technological characterization of selected LAB isolates

The strains identified at species level were investigated for their technological potential to be used as starter cultures in chickpea fermentations. Technological analyses were performed in duplicate.

2.7.1. Acidification activity

The acidification test of the selected strains was performed in sterile flour extract (SFE) broth according to the method described by Alfonzo et al. (2016). Overnight grown LAB cultures in MRS broth were harvested by centrifugation at 13,300 rpm for 3 min (Thermo Scientific MicroCL 17, Germany), washed with sterile Ringer's solution and resuspended in the same solution to an optical density at 600 nm of 1.00 (Shimadzu UV-1700, Japan) to standardize bacterial inocula. Twenty mL of SFE was inoculated with 1% (v/v) of the solution consisting of the cell suspension and incubated at 30°C. The acidifying capacity of the LAB was monitored during their incubation at 30°C by pH measurements taken at 2-h intervals for the first 8 h of incubation and then at 24, 48, 72 h and 7 d (day) after inoculation. Uninoculated SFE was used as control tube.

Strains were also analysed for their ability to produce lactic and acetic acids after 8 h of fermentation in SFE. For that purpose, acidified SFE (aSFE) samples were analyzed through HPLC system (the procedure is explained at paragraph 2.8.2).

224 2.7.2. EPS production on agar medium

225 Selected bacteria were streaked onto MRS agar medium supplemented with 50 g/L sucrose
226 and incubated at 30 °C for 72 h. The formation of mucoid or viscous colonies on the agar was
227 considered to be EPS production (Lule et al., 2015).

228 2.7.3. Growth at different conditions

229 Selected strains were evaluated for growth at different conditions. For growth at different
230 temperatures, inoculated (1%) mMRS broths with strains (OD=1) were incubated at 15, 28,
231 37 and 45 °C for 2-7 d. For tolerance to different pH values, strains (OD=1) were inoculated
232 to mMRS broth (1%) prepared at pH 3.5, 4.5 and 6.5 with filter sterilized 5 N HCl and 2 N
233 NaOH solutions and incubated at 30 °C for 3 d. For tolerance to different salt concentrations,
234 strains (OD=1) were inoculated in mMRS broth (1%) containing 4, 6 and 8% NaCl (w/v) and
235 incubated at 30 °C for 3 d. The ability to ferment various carbohydrates was evaluated using
236 MRS broth prepared without glucose and meat extract. Filter sterilized sugar solutions (1%,
237 w/v) was added separately to MRS broth media. The control broth did not contain any
238 carbohydrate. Chlorophenol red (0.004%, w/v) was added as the indicator and conversion of
239 the color from red-purple to yellow indicated lowering of pH due to LAB growth and
240 production of lactic acid (Schillinger and Lücke, 1987).

241 2.7.4. Enzyme profile

242 The enzyme profile assessment was performed with the API ZYM enzyme (Biomerieux,
243 France) testing system according to the manufacturer's instructions using ZYM A and ZYM
244 B reagents.

245

246 *2.8. Production of experimental chickpea dough*

247 Chickpeas (*Koçbaşı* variety) were purchased from a local market and broken into a few pieces
248 for the production of chickpea liquid. Fifty grams of ground chickpeas were put into sterile

249 glass jars and mixed with 400 mL of boiled and cooled tap water at 37 °C. The production
250 flow diagram is shown in Fig. 1. At the end of the fermentation, chickpeas were separated and
251 the liquid was used for the production of the chickpea dough. To this purpose, chickpea liquid
252 starter and flour was mixed for dough [Dough Yield (DY) 175] production. Productions were
253 carried out in duplicate. For starter culture inoculums, the selected strain (OD= 1) was
254 inoculated at a concentration of 1% (v/w) into the chickpea liquid starter. For that purpose,
255 overnight grown culture in MRS broth were harvested by centrifugation at 13,300 rpm for 3
256 min (Thermo Scientific MicroCL 17, Germany), washed with sterile Ringer's solution and
257 resuspended in the same solution to an optical density at 600 nm of 1.00 (Shimadzu UV-1700,
258 Japan) to standardize bacterial inocula.
259 The samples were taken at the beginning (0 h) and at the end of the fermentation [18 h for
260 experimental chickpea liquid starters (ECLS), 4 h for experimental chickpea doughs (ECD)].
261 Experimental liquid starters and doughs were produced using the selected LAB strain as
262 mono-culture which was inoculated to the experimental ECLS at the beginning of
263 fermentation as shown in Fig. 1. After 18 h at 37 °C, ECLS was used for the production of the
264 experimental ECD. ECLS-0 was the unfermented chickpea liquid. ECLS-C-18 and ECLS-W-
265 18 were the fermented ECLS without inoculation and with inoculation of the selected LAB
266 starter culture, respectively. ECD samples were coded as ECD-C-0 and ECD-C-4 for the
267 control chickpea dough at 0 and 4 h of the fermentation and for the chickpea dough produced
268 with inoculated ECLS as ECD-W-0 and ECD-W-4 at 0 and 4 h of the fermentation,
269 respectively.
270
271 2.8.1. Microbiological analysis
272 Cell suspensions of ECD and ECLS experimental samples were analyzed by plate count for
273 the enumeration of the following microbial groups: mesophilic LAB on mMRS as reported

274 above; yeasts and moulds on yeast peptone dextrose (YPD) added with chloramphenicol (0.1
275 g/L), incubated aerobically for 48 h and 7 d, respectively at 28 °C; spore-forming aerobic,
276 most probably *Bacillus* spp., bacteria were investigated after heating the cell suspensions at 80
277 °C for 10 min and then spread plated on nutrient agar (NA) before aerobic incubation was
278 carried out at 37 °C for 18 h (Erginkaya et al., 2016; Halkman, 2005; Hatzikamari et al.,
279 2007b). Results were expressed as log CFU/ml or g.
280 For coliform group bacteria and presumptive *Escherichia coli*, Lauryl Sulfate Tryptose (LST)
281 liquid medium (broth) (Merck) was preferred (Clesceri et al., 1998) to conduct the Most
282 Probable Number (MPN) method which is a standard method of United States Food & Drug
283 Administration (FDA, 2002). After 24 h of incubation at 37 °C, growth and gas production in
284 the tubes indicated the presence of presumptive coliforms and these tubes were recorded as
285 positive. Gas-negative tubes were re-incubated and examined again at 48 h (Feng et al.,
286 2002). The indole test was conducted by adding 0.2-0.3 mL of Kovacs' indole reagent
287 (Merck) to the gas-positive tubes and development of a distinct red color in the upper layer
288 was recorded as positive showing the growth of an indole positive culture. Indole-positive
289 tubes were reported as presumptive *Escherichia coli* and evaluated using the MPN method
290 (Halkman, 2005).
291
292 <u>2.8.2. Chemical analysis</u>
293 Total Titratable Acidity (TTA) of the samples was determined after homogenization of 10 g
294 of sample with 90 mL of distilled water on a magnetic plate stirrer. The mixture was then
295 titrated with 0.1 N NaOH to a final pH of 8.5. TTA was expressed as the amount (mL) of 0.1
296 M NaOH needed to achieve the pH of 8.5 (Lopez et al., 2001). The pH measurements were
297 performed using a digital glass pH meter (Mettler Toledo, SevenCompact™ pH Ion S220,
298 Switzerland) by inserting the probe into the mixture (Lopez et al., 2001).

299 Ten grams of sample was homogenized with 90 mL of 25 mM phosphate buffer (pH 5.6)
300 according to the extraction method of Paramithiotis et al. (2006) and maltose, sucrose,
301 glucose, fructose, ethanol, lactic and acetic acids were determined in the extracts through
302 HPLC system consisted of a refractive index detector (RID-10A) for sugar and ethanol
303 analysis and a UV/Vis detector (SPD-20A) monitored at 210 nm for the analysis of organic
304 acids. Chromatographic separation was performed using an Aminex HPX-87H column (300 x
305 7.8 mm, Bio-Rad, Hercules, CA, USA) under the following conditions: flow rate 0.5 mL/min
306 and column temperature 50 °C. The mobile phase was 5 mM H_2SO_4. Specific Shimadzu
307 software was used for data evaluation. Stock standard solutions were prepared individually
308 from HPLC grade standards obtained from Sigma-Aldrich. Seven-point standard curves were
309 constructed from standard solutions. The limit of detection (LOD) and Limit of Quantification
310 (LOQ) values were estimated as 3 and 10 times the standard deviation derived from analysis
311 of 10 injections at the lowest calibration levels, respectively. For the recovery test, the dough
312 sample was spiked with standards during the homogenization step at final concentrations in
313 the linear range of the calibration curves. Spiked and unspiked samples of the dough were
314 analyzed under the same conditions.

315

316 2.8.3. Volatile organic compound composition

317 Volatile organic compounds (VOCs) generation in experimental samples was examined
318 according to the method of Settanni et al. (2013) with some modifications. The solid phase
319 micro extraction (SPME) technique was used with the SPME fiber (85 µm Carboxen\PDMS)
320 and GC/MS system (Agilent 7000 Series Triple Quad) equipped with an HP - 5MS capillary
321 column (30 m, 0.250 mm i.d., film thickness 0.25 mm, %5 phenyl methyl poly siloxane).
322 Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. Ionizing energy was

323 70 eV and MS was at the full-scan mode with scan range of 50–600 m/z. The identification of
324 VOCs was achieved by using the National Institute of Standards and Technology (NIST 14L)
325 reference library and VOCs were expressed as relative peak areas (peak area of each
326 compound/total area*100).

327

328 *2.9. Statistical univariate and explorative multivariate analysis*

329 Data of the analysis were subjected to one-way analysis of variance (ANOVA) and multiple
330 comparisons of means by *post hoc* Tukey's procedure using the Statistical Package for Social
331 Science 20.0 software (International Business Machines Corporation).

332 In addition, an explorative multivariate approach was employed to investigate the correlations
333 between the characteristics measured and the samples. Principal component analysis (PCA)
334 was performed with data obtained from chickpea liquid starter and dough samples following
335 the strategy applied by Martorana et al. (2017).

336 XLSTAT 2018 software (Addinsoft) for Microsoft Excel® was used for data processing and
337 graphics construction. Dissimilarity index calculation was carried out using Darwin (6.0.15)
338 software package.

339

340 **3. Results and Discussion**

341 *3.1. Illumina data analysis of the microbiota in the chickpea liquid starter and dough samples*

342 Sequences obtained from Illumina Sequencing were processed using QIIME2 software.
343 Distribution of the relative abundances (%) of bacterial genera identified by MySeq Illumina
344 in the chickpea liquid starter and dough samples is shown in Fig. 2. Thirteen bacterial genera
345 were detected. Although the major part of OTUs belonged to *Clostridium perfringens* species,
346 the LAB group was represented by the four main genera *Lactobacillus*, *Enterococcus*,

347 *Leuconostoc* and *Weissella* and some other unidentified LAB. In particular, dough samples
348 from A Bakery were characterized for the presence of *Lactobacillus* only during the first
349 collection, while all other doughs showed the presence of *Weissella*, reaching the highest
350 percentage in sample CD-N2. Also for liquid starters, the farm A was characterized by the
351 lowest presence of LAB, while the samples CLS-B1 and CLS-N2 displayed the highest
352 percentages of *Weissella*. Furthermore, the most relevant biodiversity within LAB group was
353 shown by the sample CLS-B2 that included *Leuconostoc* as major group followed by
354 *Enterococcus* and *Weissella*.
355
356 *3.2. Biodiversity of the LAB in chickpea fermentations*
357 The results of the plate counts on MRS showed a content of LAB in CLSs and CDs were in
358 the range of 1.60-7.18 log CFU/g and 4.30-6.89 log CFU/g, respectively. Putative LAB
359 cultures were analysed by RAPD-PCR analysis and a total of 50 strains were found associated
360 to the chickpea starter and dough samples. Based on the 16S rRNA sequence analysis, 32
361 strains were identified at the species level, while the remaining 18 strains were identified only
362 at the genus/family level (Table 1).
363 The frequence of isolation of the LAB species indicated a consistent presence of *W. confusa*
364 (47.8%), followed by *Enterococcus faecium* (22.1%) and *Weissella cibaria* (12.4%).
365 Furthermore, *Leuconostoc mesenteroides* (5.3%), *Lb. brevis* (3.5%) and *Streptococcus*
366 *lutetiensis* (2.7%) were found as minor species. Conversely, *Enterococcus lactis*, *Lb.*
367 *plantarum*, *Leuconostoc mesenteroides* subsp. *dextranium*, *Pediococcus acidilactici* and
368 *Pediococcus pentosaceus* were only isolated from 1 or 2 samples.
369 *Weissella confusa* was isolated from all collected chickpea liquid starter and dough samples,
370 except the CLS-A samples. *En. faecium* was commonly isolated from collected samples,
371 except in CD-N and CLS-N samples. *W. cibaria* was identified in A, B and N chickpea dough

372 samples. It was also identified in the CLS-B sample. Regarding the other isolated strains, *Ln.*
373 *mesenteroides* was only identified in the CD-A sample. The distribution of LAB identified at
374 the species level in chickpea liquid starter and dough samples is shown in Table 2.
375 In the present study, half of the identified strains belonged to the genus *Weissella* spp. and the
376 most frequently isolated species was *W. confusa*. The second most frequently isolated species
377 was *En. faecium.* In a study conducted in Turkey, chickpea dough was produced using
378 traditional procedure under laboratory conditions and LAB flora of chickpea fermentations
379 were identified. The species were identified as *Lc. lactis*, *Lb. brevis* and *Lb. plantarum* in the
380 chickpea liquid starter via phenotypic methods. In the chickpea dough, the same species and
381 also *Lb. pentosus* and *W. confusa* were detected (Cebi, 2009). In another study conducted in
382 Turkey, chickpea liquid starters and doughs collected from bakeries were investigated for
383 their microbiota and *En. casseliflavus*, *En. gallinarum*, *En. mundtii, Lb. pentosus*, *Lb.*
384 *plantarum*, *Lb. bifermentans*, *Lb. sanfranciscensis*, *Lb. viridescens*, *P. urinaeequi, S.*
385 *thermophilus* and *Lc. lactis* subsp. *cremoris* were identified (Hancıoglu-Sıkılı, 2003). The
386 species *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Pediococcus* spp. were previously
387 reported in chickpea-containing fermented foods made in India (Reddy et al., 1982).
388 Katsaboxakis and Mallidis (1996) isolated species belonging to *Lactobacillus,*
389 *Corynebacterium, Micrococcus, Pediococcus, Bacillus* and *Clostridium* genera during the
390 fermentation of coarsely ground chickpeas in water at 32, 37 and 42 °C in Greece. Saez and
391 others (2018) reported occurence of *En. durans, En. mundtii, Lc. garvieae, P. pentosaceus, W.*
392 *cibaria* and *W. paramesenteroides* in the chickpea sourdoughs produced by chickpea flour in
393 northwestern Argentina.
394 According to the results of the present study, non-*Lactobacillus* spp. dominated the chickpea
395 fermentations. The chickpea dough is characterized by a higher pH in the range of 4.82-5.67
396 (data not shown). *Lactobacillus* spp. are more resistant to acidic conditions than other LAB

397 (Hammes and Hertel, 2009). Therefore, other species that grow at higher pH values are
398 commonly identified in chickpea fermentations. In addition, chickpea fermentations are
399 conducted in a very hot environment (more than 40°C). The range of pH conditions for
400 *Weissella* spp. growth is 5-7 and they can grow up to 42-45 °C (Fusco et al., 2015).
401 *Enterococcus* species can survive temperatures above 60 °C for short periods (around 30
402 min), whereas the optimum temperature is 37 °C for *Enterococcus* and *Streptococcus* (Švec
403 and Franz, 2014). *Leuconostoc* species are non-acidophilic and the optimal temperature for
404 their growth is in the range of 10-37 °C (Pikuta and Hoover, 2014).
405
406 *3.3. Evaluation of the technological attributes of selected LAB in chickpea fermentations*
407 Members of most frequently isolated *W. confusa* species isolated investigated for their
408 fermentative potential. For this reason, the strains *W. confusa* RL425, RL1139, RL898,
409 RL910, RL1252 and BL1406 strains were subjected to the acidification test according to the
410 previously reported method of Alfonzo and others (2016) and results are shown in Table 3.
411 All selected cultures, except *W. confusa* BL1406, decreased SFE pH below 5.0 after 8 h. At
412 24 h, almost all of the strains acidified the medium below pH 4.0. The slowest acidifier strains
413 were *W. confusa* RL425, RL1252 and RL1139.
414 After 8 h of fermentation, the acidified SFE samples were also analyzed for their lactic and
415 acetic acid content. The acetic acid levels were also higher than those of previously reported
416 studies, which could be related to the composition of the flour extract. In the present study,
417 the supernatant of the flour extract was in the semi-solid form, therefore the dry matter
418 composition could be higher than the SFE taken as liquid supernatant. When the dry matter
419 content is high, it contains more carbohydrates as dry matter basis. Therefore, enough carbon
420 source for LAB to utilize can affect the organic acid production. Alfonzo et al. (2013)
421 reported the highest acetic acid content as 0.11 mg/g in the SFE inoculated with a *Weissella*

422 spp. Settanni et al. (2013) reported the lactic and acetic acid contents produced by different
423 LAB strains in sourdoughs processed with non-sterile flour in the range of 1.36-6.47 and
424 0.15-1.08 mg/g after 8 h of fermentation, respectively.

425 The strains *W. confusa* RL1139, RL1252 and RL425 showed EPS production as observed on
426 the plates. Among these strains, the mucoid texture was easily observed by a loop in the plates
427 of RL1139 and RL1252 strains as compared to RL425. Quantitative analysis was not
428 conducted for the EPS level in our study. Figure 3 shows the EPS produced plate belong to
429 RL1139 strain. Dextran production from sucrose by some *W. confusa* strains has been
430 reported previously (Björkroth et al., 2014; Collins et al., 1993; Katina et al., 2009; Lim et al.,
431 2018).

432 Based on lactic and acetic acids production and EPS generation, *W. confusa* RL1139 was
433 selected as starter culture for the experimental chickpea fermentations. Also, this strain was
434 evaluated for the growth under different conditions and for its enzyme profile.

435 *W. confusa* RL1139 grew at all temperatures tested (Table 4). It was reported that, the growth
436 of *W. confusa* at 45 °C is strain dependent and some strains showing good growth at this
437 temperature (Collins et al., 1993). *Weissella confusa* RL1139 grew at 4% NaCl and pH 4.5
438 and 6.5. However, it did not grow in the presence of 6 and 8% NaCl and at pH 3.5. *W.*
439 *confusa* RL1139 fermented glucose, fructose, sucrose, maltose, mannose and xylose. Acid
440 production from xylose, but not from arabinose, lactose, and raffinose was also reported for
441 *W. confusa* strains by Fusco et al. (2015). Regarding the enzyme pattern of *W. confusa*
442 RL1139 it was characterized by alkaline phosphatase, acid phosphatase and naphthol-AS-Bi-
443 phosphohydrolase activities. Some *Weissella* species were reported to produce those enzymes
444 in other studies. In the study of Kang and others (2019), *W. cibaria* strains showed positive
445 results for acid phosphatase and naphthol-AS-BI-phosphohydrolase enzymatic reactions
446 (Kang et al. 2019). Also, alkaline phosphatase, acid phosphatase and naphthol-AS-BI-

447 phosphohydrolase activities were reported by *W. ceti* (Vela et al., 2011). Acid phosphatases
448 hydrolyze phosphate esters (Gandhi and Chandra, 2012) and alkaline phosphatase catalyzes
449 the hydrolysis of phosphate monoesters at a high pH (Sharma et al., 2014). It was reported
450 that bacterial non-specific acid phosphohydrolases or phosphatases are physiologically help
451 the cell to utilize the organic phosphoesters that cannot cross the cytoplasmic membrane thus
452 providing the cell with essential nutrients (Gandhi and Chandra, 2012).
453
454 *3.4. Characteristics of experimental dough samples*
455 The values of pH and TTA registered for the ECLS and ECD are shown in Table 5. The initial
456 pH of control and inoculated chickpea liquid starters were 6.93 and 6.95, respectively which
457 reached the values of 4.82 and 4.92, respectively, after 18 h of fermentation. The final pH
458 values of the control and inoculated chickpea doughs were 4.82 and 4.79, respectively. The
459 pH of chickpea liquid fermentation during the first 10 h are reported in Fig. 4. According to
460 the results, pH started to decrease after 6 h in the control liquid. Conversely, the pH of the
461 inoculated liquid decreased after 2 h.
462 After 18 h, the TTA of the control and inoculated liquid starter samples were 4.40 and 4.10
463 mL 0.1 N NaOH/10 g sample, respectively. Final TTA values of the control and inoculated
464 doughs produced were 5.30 and 6.00 mL 0.1 N NaOH/10 g sample, respectively.
465 Hancıoglu-Sıkılı (2003) used three different starter cultures, *Lc. lactis* subsp. *cremoris*, *Lb.*
466 *bifermantas* and *Lb. viridescens,* as mono-cultures for the production of chickpea liquid
467 starter. Higher acidification was detected in the dough samples produced by *Lactabacillus*
468 spp. than produced *Lactoccocus* spp. Final pH and TTA values were in the range of 4.91-5.25
469 and 0.41-0.74 %, respectively (Hancıoglu-Sıkılı, 2003). In another study, chickpea
470 fermentations were conducted with three different LAB cultures (*Lb. brevis* FK2, *Lc. lactis*
471 FK5 and *Lb. plantarum* FK25) and the pH values of the chickpea doughs were determined in

472 the range of 4.83-4.92. It was reported that differences in the pH values of the chickpea
473 doughs were not significant and spontaneous flora in the chickpea fermentations could affect
474 the final pH values (Cebi, 2014). Reported acidity values were in accordance with the present
475 study.

476 The microbial loads of the ECLS and ECD samples are reported in Table 6. Chickpea liquid
477 starter was inoculated with the culture at 6 log CFU/ml; hence, LAB counts of the inoculated
478 sample was around 6-7 log CFU/ml. On the other hand, presumptive LAB counts of the
479 control liquid were very low. The cell counts of both liquid starters on the mMRS were
480 increased at the end of the 18 h fermentation; however, increase in the inoculated liquid
481 starter was higher than the control liquid starter. Cell counts on mMRS agar were higher in
482 the chickpea dough produced with the inoculated chickpea liquid starter compared with
483 control dough. Final LAB counts were 9.56 and 11.01 log CFU/g in the ECD-C and ECD-W,
484 respectively. Presumptive yeast counts varied during fermentation. Yeast counts were 3.00
485 and 4.86 log CFU/g in the dough samples at the end of the fermentation, respectively.

486 At the end of the fermentation, aerobic spore forming bacteria, most probably *Bacillus* spp.,
487 were 4.60, 3.65, 5.00 and 4.70 log CFU/mL or g in the ECLS-C, ECLS-W, ECD-C and ECD-
488 W, respectively. *Bacillus* spp. was reported as the dominant microbiota in chickpea liquids
489 previously (Hatzikamari et al., 2007b). Moulds were detectable only in sample ECD-C-0.
490 When present, coliforms were represented by *Escherichia coli*.

491 The contents of carbohydrate and organic acid in the experimental chickpea liquid starter and
492 dough samples are given in Table 7. The results of ethanol are not reported in table, because
493 no sample was scored as positive. ECLS samples representing the beginning of the
494 fermentation were taken directly from the water after mixed with chickpeas; hence,
495 compounds in the chickpeas could not be passed into the water yet. Therefore, all of the
496 detected compounds were <LOQ in the liquid at the beginning of the fermentation. At the end

497 of the chickpea liquid fermentation, differences in the maltose+sucrose and glucose contents
498 were not significant between the liquid starters produced with and without starter culture.
499 Lactic acid production was higher in the control liquid starter than inoculated liquid. This can
500 be related to the spontaneous flora present in the chickpea liquid. Differences in the
501 maltose+sucrose and lactic acid contents were significant but glucose, fructose and acetic acid
502 contents were not significant between the doughs produced with and without starter culture.
503 Acetic acid was produced more than lactic acid in the doughs. Ethanol contents of all samples
504 determined <LOQ. The possible explanation can be low yeast counts in the samples or
505 evaporation of ethanol during the sampling. In the producton of chickpea dough, flour is
506 added and hydrolysis of the carbohydrates by flour enzymes can affect sugar content in the
507 dough besides bacterial consumption (Paramithiotis et al., 2006; Hansen, 2012). In the present
508 study, sugars were not completely consumed during 4 hours. Sugars levels in the samples can
509 decrease and increase as a result of bacterial consumption and hydrolisation by flour enzymes,
510 respectively. Therefore, it is difficult to discuss the consumption ratio of sugars by
511 microorganisms. It has been reported previously that carbohydrates are continuously liberated
512 during fermentation, especially by endogenous flour enzymes, and it was not possible to
513 estimate their consumption rate (Lattanzi et al., 2013).
514 The SPME-GC-MS chromatographic analysis revealed the presence of 21 VOC compounds
515 in experimental chickpea fermentations (Table 8). VOC compounds were determined based
516 on the relative peak area. In unfermented chickpea liquid starter at 0 h, only acetaldehyde and
517 ethenyl formate were detected. Unfermented chickpea liquid only contains chickpea seeds and
518 water and at the 0th hour of the fermentation, any microbial activity does not exist. Therefore,
519 lower number of VOCs were detected. In the fermented ECLS and ECD samples, butanoic
520 acid (synonym butyric acid) showed the highest relative area. Relative peak area of butanoic
521 acid was 74.26 and 81.52 %, in the control and adapted dough produced with inoculated

522 chickpea liquid starter, respectively. In chickpea fermentations, production of butyric acid can
523 be related to the presence of *Clostridium* species as reported previously (Katsaboxakis and
524 Mallidis, 1996), because some strains of *Clostridium* spp. produce butyric acid (He et al.,
525 2005; Yang et al., 2011). Hancıoglu-Sıkılı (2003) reported the occurence of butanoic and
526 acetic acid acid in the chickpea liquid starter and dough samples produced with various starter
527 cultures. Cebi (2014) investigated the volatile profile of the chickpea dough and bread
528 samples produced with different starter cultures and 1% baker's yeast and determined
529 alcohols including ethanol, 1-butanol, 1-hexanol, 1-octan-3-ol, aldehydes including hexanal
530 and acetaldehyde, esters including ethyl acetate and hexyl butanoate more than other
531 compounds in chickpea dough samples. In the present study, relative peak areas of butyl
532 butanoate and ethyl butanoate were higher than other VOCs in fermented chickpea liquid
533 starters. Relative area of butanoic acid was very high in the fermented chickpea dough
534 samples and other VOCs were determined at low levels compared to butanoic acid. In the
535 control dough, acetic acid, acetone, butyl acetate and butyl butanoate were other detected
536 VOCs. On the other hand, in the fermented dough produced by inoculated chickpea liquid
537 starter, acetic acid, butyl acetate and ethyl acetate peaks were determined.

538

539 *3.5. Multivariate statistical analysis*

540 Totally 31 variables were expressed as linear combination of the first two factors (F1 and F2).
541 The variables were grouped as microbiological, chemical and VOC compounds and coded as
542 M, C and V letters, respectively. The score and loading plots of PCA analysis (Fig. 5 A-B)
543 shows that an overall 58.45% of variance was explained by the first component (F1 of
544 36.31%) and second component (F2 of 22.14%).

545 According to the plot (Fig 5), unfermented chickpea liquid differed from the dough samples
546 with regards to F1 and from the chickpea liquid starters with regards to F2. Along with F1,

547 fermented chickpea liquid starters and dough samples were separated from each other.
548 Control and inoculated samples were close to each other.
549 As it can be seen, both control and *W. confusa* added chickpea liquid starter and dough
550 samples were close to each other in the plot. It is important to protect typical characteristics of
551 the chickpea dough in terms of aroma and taste. Therefore, it is preferred not to change its
552 characteristics after starter addition to those kind of traditional foods,
553 Therefore, according to our results, addition of *W. confusa* selected strain did not change
554 typical chracteristics of the doughs. On the other hand, starter addition will enable chickpea
555 dough production at the same quality and characteristics everytime compared to spontaneous
556 fermentation.
557 **4. Conclusions**
558 In this study, LAB biodiversity of the chickpea fermentations were investigated and chickpea
559 dough was produced with a selected *W. confusa* strain isolated from spontaneous chickpea
560 fermentations. Different LAB species were identified in the samples while *Weissella* spp.
561 dominated LAB community. *W. confusa* constituted almost half of the identified samples. The
562 most relevant *W. confusa* strain was chosen as starter culture showing the most relevant
563 technological performance. *W. confusa* RL1139 strain was used in the experimental chickpea
564 fermentations and our results showed that starter culture addition did not change overall
565 characteristics of the produced chickpea doughs. It is an important output of the present study.
566 Because in the production of those kind of fermented foods, typical characteristics of the
567 product should be protected. Therefore, by starter culture addition, production of a typical
568 chickpea dough at the same quality everytime should be possible. Also, large scale production
569 of chickpea bread will be possible industrially by starter culture addition. In the starter
570 selection, it is important to choose according to the properties of the end product. Chickpea
571 dough is generally referred as “sweet dough” in many regions and in order to reach the

572 desired level of acidification, strains showing strong acidification should not be used as starter
573 culture. In the present study, culture-independent analysis for microbiota diversity identified
574 *Clostridium perfringens* as major group in all samples. Therefore, in the future studies,
575 different lactic acid bacteria culture combinations but also *Clostridium* spp. and *Bacillus* spp.
576 can be investigated to be used as starter culture.

577

578

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587 **Authors' Contributions**

588 CPBG conceived and designed all of the analysis, collected all of the samples, performed all
589 of the laboratory analysis, conducted all of the data tools and analyses, wrote the manuscript
590 and reviewed the final paper; HE conceived and designed all of the analysis, supervised the
591 sample collection and analysis, contributed all of the data tools, contributed to writing the
592 manuscript and reviewed the final paper; LS contributed data tools, performed Illumina data
593 analysis, contributed to writing the manuscript and reviewed the final paper; EF performed
594 Illumina data analysis and wrote methods and results for the Illumina data; RG extracted and
595 evaluated DNA quality for Illumina data analysis and reviewed the final paper.

596 **Conflicts of Interest**

597 The authors declare that there is no conflict of interest.

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767 **Table 1** Identification of the LAB isolated from chickpea liquid starters and doughs

Strain	Source	Family/Genus/Species	BLAST homology (%)	Sequence length (bp)	Acc. No.
RL1189	CD-A	*Enterococcaceae*	86 (NR_114742.1)	1369	MH704237
XL486	CD-A	*Enterococcus* spp.	95 (NR_114742.1)	1552	MH704227
XL493	CD-A	*Enterococcus* spp.	96 (NR_114453.1)	1490	MH704228
BL514	CD-A	*Enterococcus* spp.	94 (NR_114453.1)	1458	MH704231
XL880	CD-N	*Enterococcus* spp.	97 (NR_114742.1)	1478	MH704232
RL1137	CD-B	*Enterococcus* spp.	97 (NR_114742.1)	1477	MH704235
XL1199	CD-A	*Enterococcus* spp.	94 (NR_114742.1)	1562	MH704238
XL484	CD-A	*En. faecium*	98 (NR_114742.1)	1412	MH704148
XL1150	CD-B	*En. faecium*	98 (NR_114742.1)	1413	MH704158
BL1171	CLS-B	*En. faecium*	98 (NR_114742.1)	1424	MH704161
RL1184	CD-A	*En. faecium*	98 (NR_114742.1)	1477	MH704162
RL1223	CLS-A	*En. faecium*	98 (NR_114742.1)	1522	MH704165
RL1227	CLS-A	*En. faecium*	98 (NR_114742.1)	1580	MH704166
BL1229	CLS-A	*En. lactis*	98 (NR_117562.1)	1559	MH704167
RL1133	CD-B	*Lactobacillaceae*	94 (NR_042057.1)	1453	MH704234
RL1158	CLS-B	*Lactobacillus* spp.	96 (NR_114251.1)	1485	MH704236
BL1363	CD-N	*Lactobacillus* spp.	95 (NR_117814.1)	1487	MH704239
RL1165	CLS-B	*Lb. brevis*	98 (NR_116238.1)	1407	MH704159
RL1169	CLS-B	*Lb. brevis*	98 (NR_116238.1)	1482	MH704160
BL1233	CLS-A	*Lb. brevis*	98 (NR_116238.1)	1489	MH704168
BL1196	CD-A	*Lb. plantarum*	98 (NR_113338.1)	1420	MH704163
BL509	CD-A	*Ln. mesenteroides*	98 (NR_074957.1)	1466	MH704149
BL513	CD-A	*Ln. mesenteroides*	98 (NR_074957.1)	1480	MH704151
RL1253	CLS-B	*Ln. mesenteroides* subsp. *dextranium*	98 (NR_040817.1)	1508	MH704170
RL453	CLS-B	*P. acidilactici*	98 (NR_042057.1)	1510	MH704146
RL1220	CLS-A	*P. acidilactici*	98 (NR_042057.1)	1538	MH704164
BL512	CD-A	*P. pentosaceus*	98 (NR_042058.1)	1467	MH704150
XL890	CD-N	*Streptococcaceae*	94 (NR_040956.1)	1424	MH704233
BL1367	CD-N	*Streptococcus* spp.	95 (NR_115719.1)	1507	MH704240
XL1377	CD-N	*Streptococcus* spp.	95 (NR_115719.1)	1527	MH704242
RL1387	CLS-N	*Streptococcus* spp.	98 (NR_115719.1)	1368	MH704243
XL1400	CLS-N	*Streptococcus* spp.	95 (NR_042051.1)	1555	MH704244
RL1346	CD-N	*S. lutetiensis*	99 (NR_115719.1)	1496	MH704171
BL1362	CD-N	*S. lutetiensis*	98 (NR_042051.1)	1494	MH704173
RL1386	CLS-N	*S. lutetiensis*	98 (NR_042051.1)	1591	MH704174
RL498	CD-A	*Weissella* spp.	95 (NR_113258.1)	1525	MH704229
BL504	CD-A	*Weissella* spp.	96 (NR_113258.1)	1465	MH704230
XL1368	CD-N	*Weissella* spp.	98 (NR_113258.1)	1335	MH704241
RL458	CLS-B	*W. cibaria*	98 (NR_036924.1)	1446	MH704147
RL899	CD-N	*W. cibaria*	98 (NR_036924.1)	1512	MH704153
BL1361	CD-N	*W. cibaria*	98 (NR_036924.1)	1540	MH704172
RL419	CD-B	*W. confusa*	98 (NR_113258.1)	1526	MH704144
RL425	CD-B	*W. confusa*	98 (NR_113258.1)	1461	MH704145
RL898	CD-N	*W. confusa*	98 (NR_113258.1)	1476	MH704152
RL900	CD-N	*W. confusa*	99 (NR_113258.1)	1523	MH704154
RL902	CD-N	*W. confusa*	98 (NR_113258.1)	1552	MH704155
RL910	CD-N	*W. confusa*	98 (NR_113258.1)	1455	MH704156
RL1139	CD-B	*W. confusa*	98 (NR_113258.1)	1481	MH704157
RL1252	CLS-B	*W. confusa*	98 (NR_113258.1)	1487	MH704169
BL1406	CLS-N	*W. confusa*	98 (NR_113258.1)	1533	MH704175

768 Abbreviations: *En.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Ln.*, *Leuconostoc*; *P.*, *Pediococcus*; *S.*, *Streptococcus*; *W., Weissella*; Acc. No.,
769 Accession Number; CD-A, chickpea dough bakery A; CD-B, chickpea dough bakery B; CD-N, chickpea dough bakery N; CLS-A, chickpea
770 liquid starter bakery A; CLS-B, chickpea liquid starter bakery B; CLS-N, chickpea liquid starter bakery N.

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Table 2 Distribution of the isolates of the LAB species among chickpea liquid starter and dough samples.

Species	CD-A	CD-B	CD-N	CLS-A	CLS-B	CLS-N
En. faecium	8	5		7	5	
En. lactis				1		
Lb. brevis				2	2	
Lb. plantarum	1			1		
Ln. mesenteroides	6					
Ln. mesenteroides subsp. *dextranium*					1	
P. acidilactici				1	1	
P. pentosaceus	1					
S. lutetiensis			2			1
W. confusa	1	21	18		13	1
W. cibaria	3	3	2		6	
Total	20	29	22	12	28	2

Abbreviations: *En.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Ln.*, *Leuconostoc*; *P.*, *Pediococcus*; *S.*, *Streptococcus*; *W., Weissella*; CD-A, chickpea dough bakery A; CD-B, chickpea dough bakery B; CD-N, chickpea dough bakery N; CLS-A, chickpea liquid starter bakery A; CLS-B, chickpea liquid starter bakery B; CLS-N, chickpea liquid starter bakery N.

Table 3 Kinetics of acidification and organic acids produced in sterile flour extract broth by LAB strains.

Species	Strain	pH									Lactic acid (mM)	Acetic acid (mM)
		0 h	2 h	4 h	6 h	8 h	24 h	48 h	72 h	7d		
W. confusa	BL1406	6.13 ± 0.03^{ab}	6.06 ± 0.01^{a}	5.87 ± 0.04^{a}	5.58 ± 0.03^{a}	5.29 ± 0.00^{a}	3.80 ± 0.02^{b}	3.71 ± 0.01^{b}	3.62 ± 0.02^{a}	3.36 ± 0.03^{a}	7.26 ± 0.09^{d}	17.39 ± 0.15^{c}
W. confusa	RL1139	6.02 ± 0.01^{cd}	5.82 ± 0.00^{c}	5.28 ± 0.00^{d}	4.71 ± 0.02^{d}	4.49 ± 0.04^{b}	3.94 ± 0.00^{a}	3.65 ± 0.03^{b}	3.51 ± 0.04^{b}	3.34 ± 0.02^{a}	10.14 ± 0.10^{a}	26.45 ± 0.26^{a}
W. confusa	RL1252	5.97 ± 0.01^{d}	5.67 ± 0.03^{d}	5.09 ± 0.00^{e}	4.56 ± 0.02^{e}	4.30 ± 0.01^{c}	3.98 ± 0.03^{a}	3.81 ± 0.00^{a}	3.63 ± 0.01^{a}	3.30 ± 0.01^{ab}	9.04 ± 0.15^{b}	18.26 ± 0.13^{b}
W. confusa	RL425	6.17 ± 0.04^{a}	6.00 ± 0.02^{b}	5.42 ± 0.02^{c}	4.79 ± 0.01^{c}	4.48 ± 0.03^{b}	3.99 ± 0.03^{a}	3.86 ± 0.04^{a}	3.65 ± 0.03^{a}	3.26 ± 0.00^{b}	7.79 ± 0.21^{c}	17.63 ± 0.09^{c}
W. confusa	RL898	5.99 ± 0.04^{d}	5.55 ± 0.02^{e}	4.72 ± 0.02^{f}	4.32 ± 0.03^{f}	4.13 ± 0.01^{d}	3.66 ± 0.01^{c}	3.52 ± 0.03^{c}	3.47 ± 0.00^{b}	3.36 ± 0.04^{a}	8.78 ± 0.13^{b}	14.03 ± 0.12^{d}
W. confusa	RL910	6.08 ± 0.01^{bc}	5.95 ± 0.03^{b}	5.55 ± 0.03^{b}	5.16 ± 0.03^{b}	4.48 ± 0.03^{b}	3.68 ± 0.03^{c}	3.49 ± 0.03^{c}	3.40 ± 0.00^{c}	3.33 ± 0.03^{a}	7.23 ± 0.17^{d}	12.20 ± 0.21^{e}
Statistical significance[a]		***	***	***	***	***	***	***	***	**	***	

Abbreviation: *W.*, *Weissella*.

[a]Data within a column followed by the same letter are not significantly different according to Tukey's test. P value: ** $P<0.01$; *** $P<0.001$.

1 **Table 4** Growth characteristics of *W. confusa* RL1139 at different conditions

Characteristic	Growth
Growth at	
15°C	+
28°C	+
37°C	+
45°C	+
%4 NaCl	+
%6 NaCl	-
%8 NaCl	-
pH 3.5	-
pH 4.5	+
pH 6.5	+
Hydrolysis of	
Glucose	+
Fructose	+
Sucrose	+
Maltose	+
Galactose	-
Lactose	-
Mannose	+
Mannitol	-
Raffinose	-
Xylose	+
Ramnose	-
Arabinose	-
Activity of	
Alkaline phosphatase	+
Esterase	-
Esterase Lipase	-
Lipase	-
Leucine arylamidase	-
Valine arylamidase	-
Cystine arylamidase	-
Trypsin	-
α-chymotrypsin	-
Acid phosphatase	+
Naphthol-AS-Bi-phosphohydrolase	+
α -galactosidase	-
β -galactosidase	-
β -glucuronidase	-
α -glucosidase	-
β - glucosidase	-
N-acetyl- β- glucosaminidase	-
α -mannosidase	-
α -fucosidase	-

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Table 5 Kinetics of acidification during experimental chickpea liquid starter and chickpea dough fermentations

Samples	pH *(Variable C4)*			TTA[*] *(Variable C5)*		
	0 h	4 h	18 h	0 h	4 h	18 h
ECLS-C	6.93 ± 0.02[a]	n.d.	4.82 ± 0.01[b]	0.00 ± 0.00[a]	n.d.	4.40 ± 0.10[a]
ECLS-W	6.95 ± 0.01[a]	n.d.	4.92 ± 0.01[a]	0.00 ± 0.00[a]	n.d.	4.10 ± 0.00[b]
Statistical significance[**]	N.S.		***	N.S.		**
ECD-C	5.38 ± 0.03[b]	4.82 ± 0.02[a]	n.d.	3.20 ± 0.10[a]	5.30 ± 0.10[b]	n.d.
ECD-W	5.44 ± 0.01[a]	4.79 ± 0.02[a]	n.d.	3.50 ± 0.20[a]	6.00 ± 0.10[a]	n.d.
Statistical significance[**]	*	N.S.		*	***	

Abbreviations: TTA, total titratable acidity; n.d. not determined.
[*]Calculated as ml of 0.1 N NaOH.
[**]Data within a column followed by the same letter are not significantly different according to Tukey's test. P value: *, $P < 0.05$ **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant.

22 **Table 6** Microbial counts during experimental chickpea liquid starter and chickpea dough
23 fermentations

Samples	LAB † *(Variable M1)*	Yeasts† *(Variable M2)*	Moulds†	Aerobic spore-forming† *(Variable M3)*	Total coliform‡	*E. coli*‡¶
ECLS-C-0	$<1^{b}$	1.30 ± 0.21^{a}	$<1^{a}$	$<1^{a}$	<0.3	0
ECLS-W-0	6.95 ± 0.31^{a}	1.30 ± 0.18^{a}	$<1^{a}$	$<1^{a}$	<0.3	0
Statistical significance*	***	N.S.	N.S.	N.S.	-	-
ECLS-C-18	5.50 ± 0.26^{b}	1.00 ± 0.11^{b}	$<1^{a}$	4.60 ± 0.23^{a}	<0.3	0
ECLS-W-18	10.89 ± 0.40^{a}	5.50 ± 0.30^{a}	$<1^{a}$	3.65 ± 0.21^{b}	<0.3	0
Statistical significance*	***	***	N.S.	**	-	-
ECD-C-0	7.39 ± 0.34^{b}	2.85 ± 0.16^{b}	2.00 ± 0.26^{a}	4.30 ± 0.18^{a}	11	3.6
ECD-W-0	8.48 ± 0.21^{a}	6.24 ± 0.25^{a}	$<1^{b}$	3.00 ± 0.20^{a}	11	3.6
Statistical significance*	**	***	***	N.S.	-	-
ECD-C-4	9.56 ± 0.33	3.00 ± 0.16^{b}	$<1^{a}$	5.00 ± 0.21^{a}	3.6	0
ECD-W-4	11.00 ± 0.36	4.86 ± 0.21^{a}	$<1^{a}$	4.70 ± 0.33^{a}	3.6	0
Statistical significance*	**	***	N.S.	N.S.	-	-

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25 Abbreviations: ECLS-C, experimental chickpea liquid starter control; ECLS-W, experimental chickpea liquid starter *Weissella*; ECD-C,
26 experimental chickpea dough control; ECD-W, experimental chickpea dough *Weissella*; LAB, Lactic acid bacteria.
27 †Units are log CFU/mL for liquid sample and log CFU/g for dough sample. Results indicate mean values ± standard deviation (SD) of four plate
28 counts (carried out in duplicate for two independent productions).
29 ‡ estimated by MPN.
30 ¶ *Escherichia coli*
31 *Data within a column followed by the same letter are not significantly different according to Tukey's test. P value: ** P<0.01; *** P<0.001; N.S.,
32 not significant.
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34 **Table 7** Carbohydrate and organic acid contents (g/kg) of the experimental chickpea liquid
35 starter and chickpea dough samples.

Samples	Carbohydrate/Organic acid				
	Maltose + sucrose *(Variable C6)*	Glucose *(Variable C7)*	Fructose *(Variable C8)*	Lactic acid *(Variable C9)*	Acetic acid *(Variable C10)*
ECLS-0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ECLS-C-18	0.30 ± 0.07[a]	0.75 ± 0.08[a]	0.34 ± 0.00[a]	1.05 ± 0.14[a]	1.29 ± 0.01[a]
ECLS-W-18	0.32 ± 0.07[a]	0.81 ± 0.01[a]	<LOQ[b]	0.70 ± 0.02[b]	1.57 ± 0.33[a]
Statistical significance[a]	N.S.	N.S.	***	*	N.S.
ECD-C-0	11.83 ± 0.29[b]	2.31 ± 0.19[b]	4.71 ± 0.99[a]	0.99 ± 0.29[a]	1.23 ± 0.07[a]
ECD-W-0	13.29± 0.25[a]	3.69± 0.38[a]	5.42± 0.49[b]	0.74± 0.03[b]	2.74 ± 0.13[b]
Statistical significance[a]	**	*	*	**	*
ECD-C-4	14.76± 0.95[a]	3.11 ± 0.33[b]	4.79 ± 0.65[a]	1.72 ± 0.63[a]	1.96 ± 0.09[b]
ECD-W-4	14.62 ± 0.58[a]	3.51 ± 0.02[a]	5.33 ± 0.27[b]	1.63 ± 0.04[a]	3.43 ± 0.09[a]
Statistical significance[a]	N.S.	**	**	N.S.	*

36 Abbreviations: LOQ, Limit of Quantification; ECLS-C, experimental chickpea liquid starters control; ECLS-W, experimental chickpea
37 liquid starters *Weissella*; ECD-C, experimental chickpea dough control; ECD-W, experimental chickpea dough *Weissella*.
38 Results indicate mean values ± SD of four determinations
39 [a]Data within a column followed by the same letter are not significantly different according to Tukey's test. P value: *, $P < 0.05$ **, $P < 0.01$;
40 ***, $P < 0.001$; N.S., not significant.
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42 **Table 8** VOCs in the experimental chickpea fermentations as relative peak area (%).

VOC compounds[a] *(Variable)*	Samples						
	ECLS-0	ECLS-C-18	ECLS-W-18	ECD-C-0	ECD-W-0	ECD-C-4	ECD-W-4
1,3-dichloro-benzene *(V11)*	n.d.	n.d.	n.d.	0.07	0.22	n.d.	n.d.
2-Nonynoic acid *(V12)*	n.d.	n.d.	n.d.	n.d.	n.d.	0.09	n.d.
2-Octynoic acid *(V13)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04
3-hydroxy-butanal *(V14)*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.09
3-methyl-butanal *(V15)*	n.d.	n.d.	0.30	n.d.	n.d.	n.d.	n.d.
3-methyl-pentanal *(V16)*	n.d.	0.38	2.31	n.d.	n.d.	n.d.	n.d.
Acetaldehyde *(V17)*	83.58	n.d.	0.19	n.d.	n.d.	n.d.	n.d.
Acetic acid *(V18)*	n.d.	n.d.	n.d.	n.d.	n.d.	5.56	4.46
Acetone *(V19)*	n.d.	10.04	9.71	9.72	n.d.	6.00	n.d.
Butanoic acid *(V20)*	n.d.	50.38	59.30	47.33	58.25	74.26	81.52
Butyl acetate *(V21)*	n.d.	3.48	n.d.	2.67	n.d.	3.81	2.30
Butyl butanoate *(V22)*	n.d.	25.83	11.91	6.34	5.12	5.81	0.89
Cyclobutanol *(V23)*	n.d.	0.34	0.79	0.20	2.05	0.23	0.24
Ethenyl acetate *(V24)*	n.d.	n.d.	n.d.	7.52	5.33	1.38	n.d.
Ethenyl formate *(V25)*	16.42	0.27	3.30	n.d.	n.d.	n.d.	0.02
Ethyl butanoate *(V26)*	n.d.	7.56	10.76	2.66	4.97	1.01	2.13
Ethyl acetate *(V27)*	n.d.	0.47	1.14	12.06	17.52	n.d.	8.15
Formyl acetate *(V28)*	n.d.	1.25	n.d.	4.01	n.d.	n.d.	n.d.
Heptanal *(V29)*	n.d.	n.d.	n.d.	0.96	n.d.	0.08	n.d.
Hexanal *(V30)*	n.d.	n.d.	0.22	5.33	5.47	1.62	0.16
Propyl-propanedioic acid *(V31)*	n.d.	n.d.	0.07	1.13	1.07	0.15	n.d.

43 Abbreviations: ECLS-C, experimental chickpea liquid starters control; ECLS-W, experimental chickpea liquid starters *Weissella*; ECD-C,
44 experimental chickpea dough control; ECD-W, experimental chickpea dough *Weissella*. 0=0-hour, 4 =4-hour, 18= 18-hour fermentation
45 [a]Results indicate mean values of two measurements and are expressed as relative peak areas (peak area of each compound/total area) × 100,
46 n.d., not detectable. The volatile organic compounds are shown alphabetically.
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72 **Legend to figures**

73 **Fig. 1.** Flowsheet of chickpea dough production. (A) control production (B) production with
74 selected strain.

75 **Fig. 2.** Distribution of the relative abundances (%) of bacterial genera identified by MySeq
76 Illumina in the chickpea liquid starter and dough samples. Abbreviations: CD-A1, chickpea
77 dough bakery A 1st collection; CD-A2, chickpea dough bakery A 2nd collection; CD-B1,
78 chickpea dough bakery B 1st collection; CD-B2, chickpea dough bakery B 2nd collection;
79 CD-N1, chickpea dough bakery N 1st collection; CD-N2, chickpea dough bakery N 2nd
80 collection; CLS-A1, chickpea liquid starter bakery A 1st collection; CLS-A2, chickpea liquid
81 starter bakery A 2nd collection; CLS-B1, chickpea liquid starter bakery B 1st collection;
82 CLS-B2, chickpea liquid starter bakery B 2nd collection; CLS-N1, chickpea liquid starter
83 bakery N 1st collection; CLS-N2, chickpea liquid starter bakery N 2nd collection.

84 **Fig. 3.** EPS production of *W. confusa* RL1139 on agar media

85 **Fig. 4.** Kinetics of acidification of control and experimental chickpea liquid starter during 10
86 h. Abbreviations: ECLS-C, experimental chickpea liquid starters control; ECLS-W,
87 experimental chickpea liquid starters *Weissella*.

88 **Fig. 5.** Score plot (A) and loading plot (B) resulting from principal component analysis on 31
89 variables including microbiological, chemical and volatile organic compounds determined on
90 experimental samples. Abbreviations: M1: MRS; M2: YPD; M3: spore forming bacteria; C4:
91 pH; C5: TTA; C6: maltose+sucrose; C7: glucose; C8: fructose; C9: lactic acid; C10: acetic
92 acid; V11: 1,3dichloro-benzene; V12: 2-Nonynoic acid; V13: 2-Octynoic acid; V14: 3-
93 hydroxy-butanal; V15, 3-methyl-butanal; V16: 3-methyl-pentanal; V17: Acetaldehyde; V18:
94 Acetic acid; V19: Acetone; V20: Butanoic acid; V21: Butyl acetate; V22: Butyl butanoate;
95 V23: Cyclobutanol; V24: Ethenyl acetate; V25: Ethenyl formate; V26: Ethyl butanoate; V27:
96 Ethyl acetate; V28: Formyl acetate; V29: Heptanal; V30, Hexanal; V31: Propyl-propanedioic
97 acid.

98 ECLS-C, experimental chickpea liquid starters control; ECLS-W, experimental chickpea
99 liquid starters *Weissella*; ECD-C, experimental chickpea dough control; ECD-W,
100 experimental chickpea dough *Weissella*.

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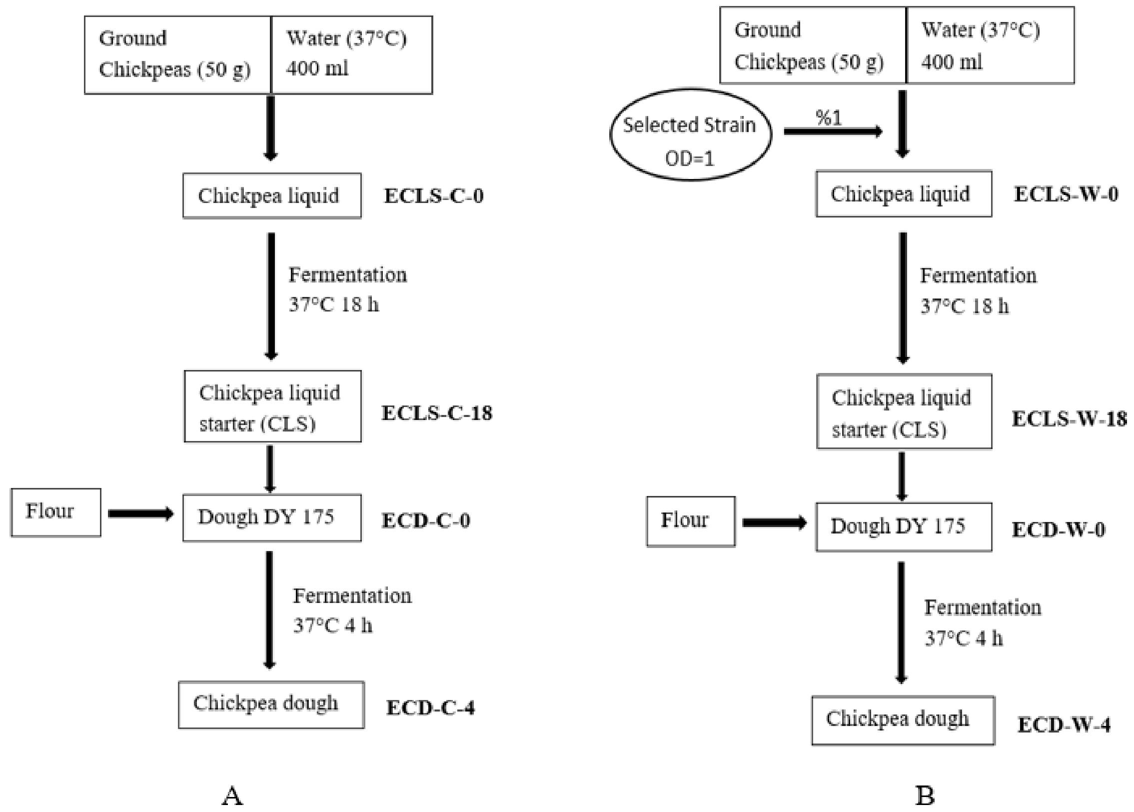
103 **Fig. 1.**



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106 **Fig. 2.**

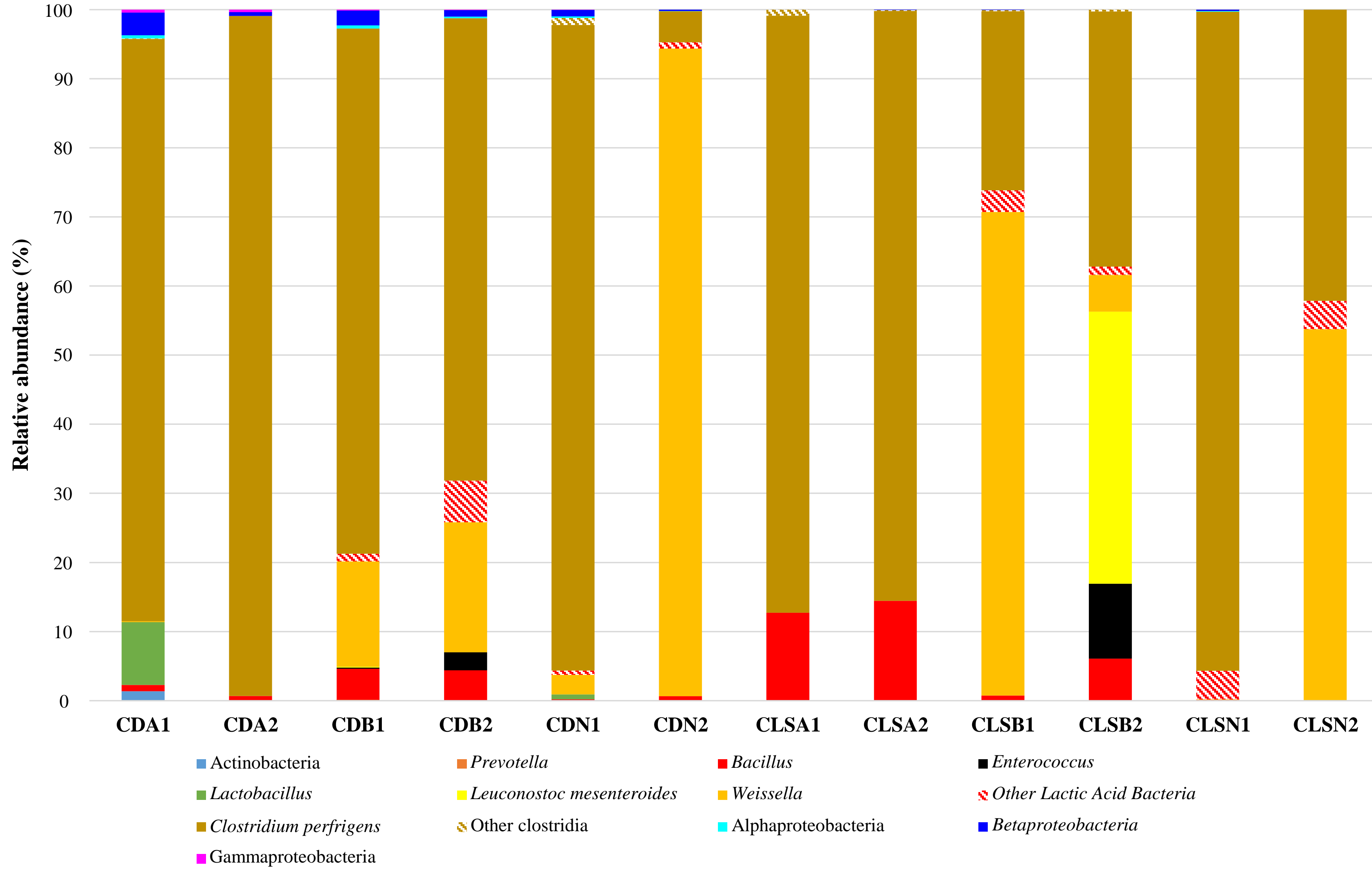

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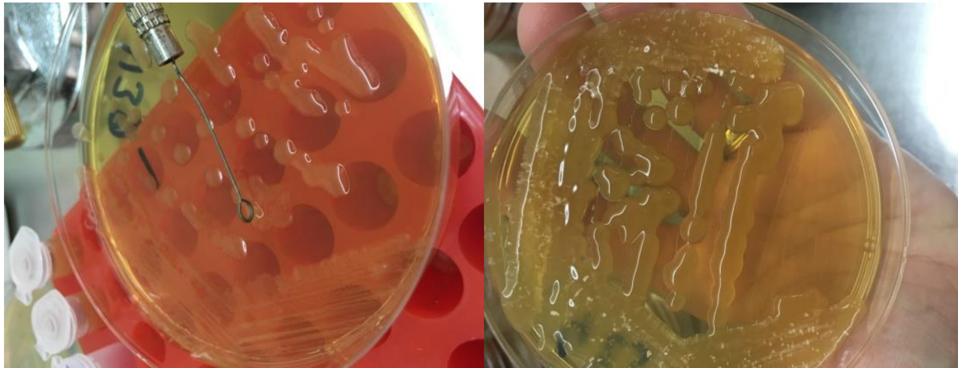
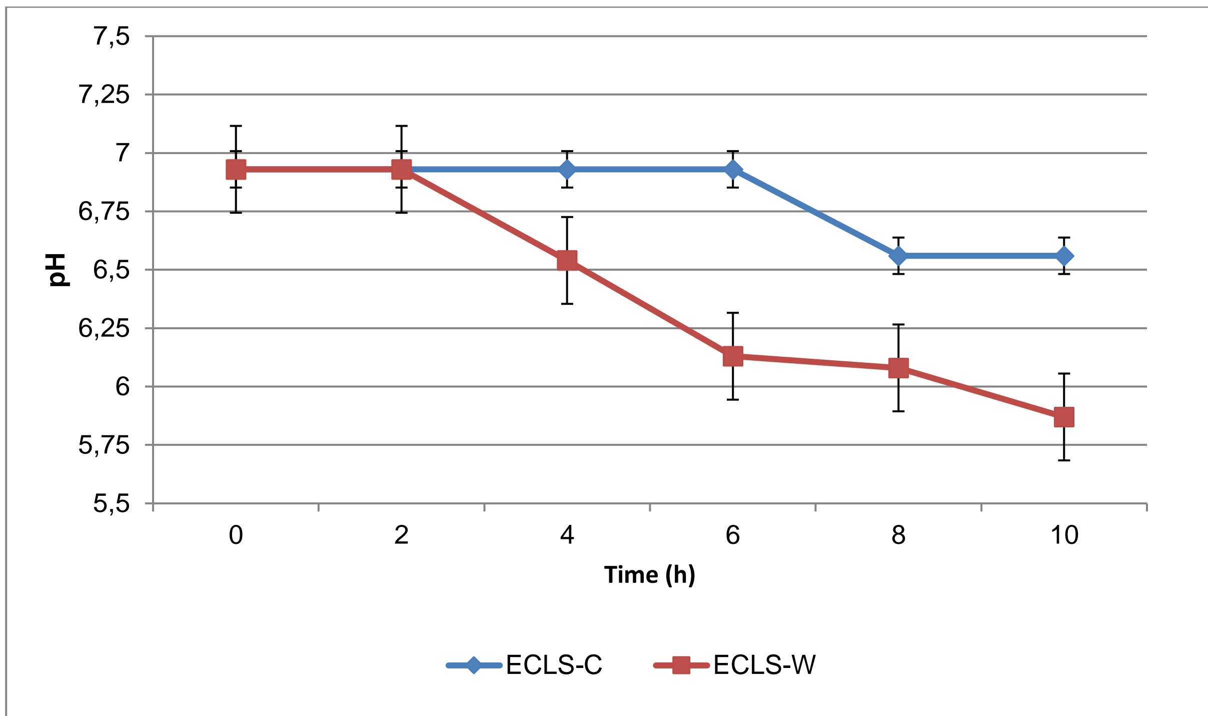
Fig 3.



130 **Fig. 4.**



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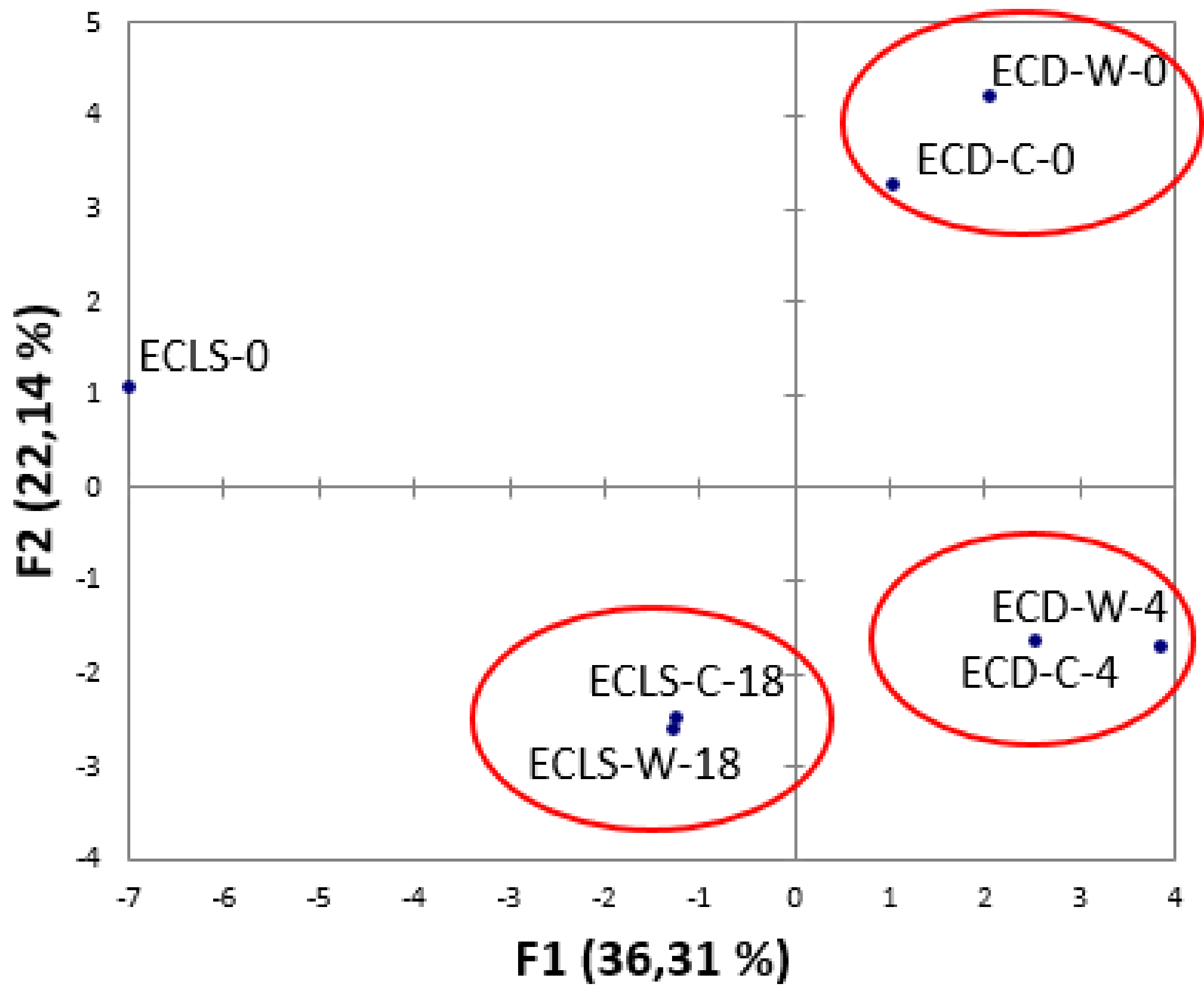
146 **Fig. 5.**



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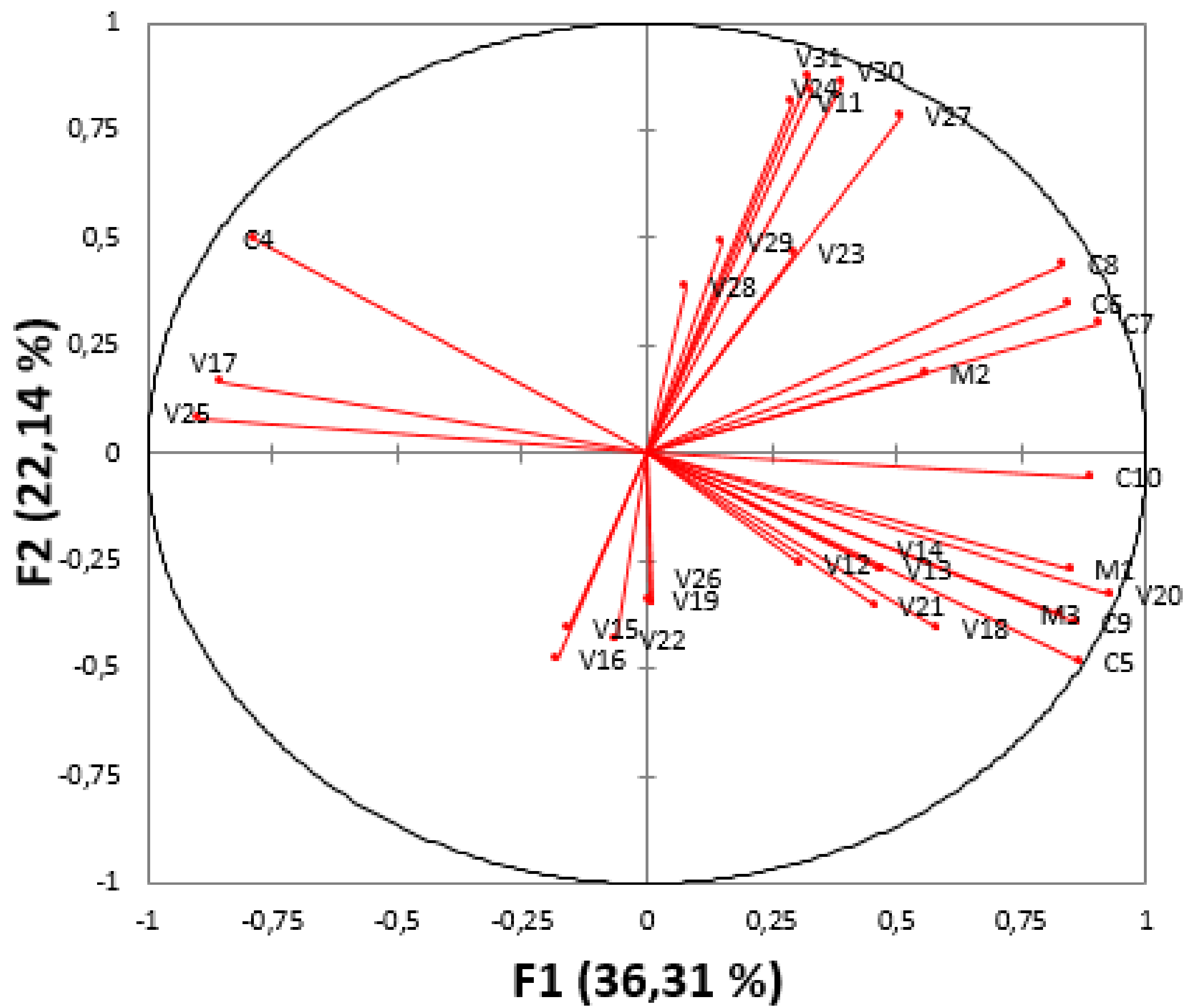
B **Variables (axes F1 and F2: 58,45 %)**



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